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Use of the Albino Guinea-pig to Detect the Skin-sensitizing Ability of Chemicals

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The guinea-pig has been used for over 20 years to demonstrate skin-sensitizing ability in chemical compounds. Correlation between the results of workers in this field is difficult because of the wide range of conditions under which tests for sensitizing potential have been performed. This has made difficult any attempt to compare the relative abilities of various chemical compounds to produce skin sensitization.

In a routine test for skin-sensitizing potential, solutions of suspected sensitizing substance have been applied over three days to the ears of guinea-pigs, and the flanks have been challenged one week later with a range of concentrations of suspected sensitizing substance. The erythematous reaction produced 24 hours after challenge was rated and compared with that in unsensitized controls. Various alternative methods of skin testing have been compared with this ear-flank test.

The ear-flank test gives good, reproducible results with many classes of chemical compound, including types of compound not previously described as giving rise to sensitization in the guinea-pig or in man, and including some compounds which are known to have carcinogenic potential. It is also demonstrated that sensitizing potential is found more frequently among aromatic (aryl) than aliphatic (alkyl) compounds. Particularly strong sensitization reactions are produced by certain aryl halides, aryl isocyanates, aryl hydrazines, *N*-nitroso compounds, and aromatic nitroso-compounds. An attempt is made to relate the results of animal tests to reported cases of human skin sensitization.

PART I

Evaluation of Skin Test Procedures

It is becoming increasingly important to industry to have available a simple, reliable, and reproducible small-animal test for potential sensitizers. Early knowledge of the sensitizing properties of new products makes it possible to instruct prospective users of these products in proper methods of handling. Similarly, early knowledge of the sensitizing properties of intermediates in chemical synthesis can, in some cases, permit replacement, before plant investment has been made, of one method of synthesis by another not involving compounds which sensitize.

An attempt to develop a single small-animal test which would be an exact model of human skin sensitization is unlikely to be successful because of the variety of forms of human skin sensitization that are known. Human skin sensitization may involve an erythematous reaction at the site of challenge or elsewhere, and this reaction may occur rapidly (immediate hypersensitivity) or it may reach maximum intensity after about 24 hours (delayed hypersensitivity).

Using the guinea-pig, it is possible to demonstrate a type of skin sensitization in which an erythematous reaction always occurs at the site of challenge and is always one of delayed hypersensitivity. Various workers have shown that the erythematous reaction in the guinea-pig is due to the presence of sensitized lymphocytes and not to soluble precipitating or reaginic antibody and can be transferred from one guinea-pig to another by transferring leucocytes (Jeter, Tremaine, and Seebohm, 1954). Skin reactions in humans may also be due to the presence of sensitized lymphocytes but, in other cases, may be due to soluble circulating reaginic antibodies. Delayed hypersensitivity reactions in guinea-pigs can be induced as quickly as five to seven days following contact with sensitizing agent, whereas most sensitizations to chemicals (Grolnick, 1960) in humans take somewhat longer (seven to 24 days)

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from first contact to develop. There are also differences in structure between guinea-pig and human skins. Despite these differences between skin sensitization in humans and in guinea-pigs, a test in guinea-pigs has considerable value because it picks out chemical substances which have the general property to sensitize, *i.e.*, it picks out substances which (*a*) penetrate the skin (highly reactive compounds would be hydrolysed by surface moisture before penetrating the skin), (*b*) after penetrating the skin are reactive with protein, and (*c*) when reacted with protein, produce antigenic conjugates.

The demonstration of skin-sensitizing power in guinea-pigs has been achieved in tests in which a variety of methods of applying sensitizing and challenge doses and a variety of intervals between these doses were used. Landsteiner and Jacobs (1936) showed that, if solutions of certain catechols were applied twice weekly for two weeks to the shaved backs of guinea-pigs and the flanks were tested three weeks later, erythema was produced. Landsteiner and Di Somma (1938) demonstrated the sensitizing action of materials such as mustard oil to guinea-pig skin in a test in which the material was applied 12 times over two weeks and then over two to three successive days three weeks later. Skin sensitization of guinea-pigs by chemical agents has also been achieved by several other procedures, *i.e.*, intraperitoneal injection (Landsteiner and Chase, 1940), intradermal injection (Landsteiner and Chase, 1937), or intraperitoneal injection of protein-hapten conjugates (Landsteiner and Chase, 1941). In all these cases an olive oil solution of the sensitizing agent was applied to the skin as a challenge three weeks later, and the erythema was rated 24 to 48 hours later. The various test procedures were reviewed at length by Chase (1954), and a description was included of a test in which olive oil solutions are applied to an area of guinea-pig flank on three successive days and challenge is carried out two weeks later. Chase (1957) also reviewed knowledge of the mechanisms involved in the sensitization of guinea-pigs to chemical agents. The increased interest in industrial laboratories in the detection of new chemical sensitizers by the use of small-animal tests is illustrated by publications such as those of Davies (1962), Buehler (1964), and Davies (1964a), and that of Hood, Neher, Reinke, and Zapp (1965) in which guinea-pig tests said to be capable of detecting weak as well as moderate and strong sensitizers are described.

To develop a test which is useful for the rapid screening for sensitizing potential of a wide range of industrial chemicals, a simple ear-flank test with guinea-pigs has been evaluated. The use of the ears for sensitization was described by Turk and Stone (1963) and used by Davies (1964b). The efficacy of the various forms of ear-flank test was compared with that of other percutaneous tests using the sensitizer 2,4-dinitrochlorobenzene (DNCB).

Method

Effect of Varying Numbers of Sensitizing Applications Each ear of six separate batches of 10 guinea-pigs was treated with 0·1 ml./day of 0·25% DNCB in olive oil for 1, 2, 3, 4, 5 or 6 days. Six challenge spots of 0·2 ml. of 0·25% DNCB in olive oil were applied to 1 cm. diameter circular areas on the clipped flanks of each animal seven days after the first application to the ears. Erythema was rated 24 hours later on an arbitrary scale of O (no erythema), Tr. (trace of erythema), ± (slight pink), + (pink), and ++ (bright pink).

Effect of Varying Time between Sensitization and Challenge Each ear of four separate batches of 10 guinea-pigs was treated once a day for three consecutive days with 0·1 ml. of 0·25% DNCB in olive oil. Six challenge spots of 0·2 ml. of 0·25% DNCB in olive oil were applied to the clipped flanks of each animal 1, 2, 3 or 4 weeks later. Erythema was rated 24 hours after challenge.

Relative Effectiveness of Sensitization via Ears and via Flanks On three consecutive days 0·1 ml. of 0·25% DNCB in olive oil was placed on each ear of 12 guinea-pigs and 0·2 ml. of 0·25% DNCB in olive oil was placed as a spot on a clipped flank of 12 other guinea-pigs. Seven days after the start of the experiment three spots of 0·2 ml. of solution were applied to a clipped flank (the unused flank in the case of the flank-sensitized animals) of each animal and the erythema was rated 24 hours later.

Effect of Continuous Application Once a day, day after day (except week-ends), 0·2 ml. of 0·25% DNCB in olive oil was applied to the centre point of a clipped area of flank. Erythema ratings were recorded just before re-application of the allergen.

Relative Effect of Varying Concentrations of Sensitizing and Challenge Solutions On three consecutive days 0·1 ml. of 0·05, 0·25 or 1·0% DNCB in olive oil was applied to each ear of batches of three guinea-pigs. Seven days after the first application to the ears spots of 0·05, 0·25, and 1·0% DNCB in olive oil were applied to the clipped flank of each animal. The erythema was rated 24 hours later.

Effect of Injections of Freund's Adjuvant Complete Freund's adjuvant (0·1 ml.) was injected into the pad of both hind feet of four guinea-pigs. Starting five days later, when the feet were fully inflamed and swollen, the four animals plus four controls were each treated on the ears for three days with 0·1 ml./day of 0·25% DNCB in olive oil. Seven days after the first application to the ears 0·2 ml. of 0·25% DNCB in olive oil was applied to the flanks. Erythemas were compared 24 hours later.

Effect of Solvent on Sensitization Each ear of separate batches of six guinea-pigs was treated for three days with 0·1 ml./day of 0·25% DNCB in test solvent. Six challenge spots of 0·2 ml. of the same solution were applied to the clipped flank of each animal seven days after the first application to the ears. Erythema was rated 24 hours after challenge.

Results

Effect of Varying Numbers of Sensitizing Applications Results (Table I) indicate that maximum sensitization is achieved by four daily applications to the ears. Results following three daily applications are only marginally inferior to those following four.

Effect of 1-, 2-, 3-, and 4-week Delays between First Sensitizing and First Challenge Doses Sensitization is seen from Table II to be greater after one week than after two weeks.

TABLE I

EFFECT ON DEGREE OF SENSITIZATION OF VARYING NUMBERS OF SENSITIZING APPLICATIONS

Animal	*Erythema Ratings* *No. of Consecutive Daily Sensitizing Applications*																					
	1			*2*			*3*				*4*				*5*				*6*			
	±	*Tr.*	0	±	*Tr.*	0	+	±	*Tr.*	0	+	±	*Tr.*	0	+	±	*Tr.*	0	+	±	*Tr.*	0
1			6			6		6				6					1	5	2	4		
2			6			6		6			2	4					6			6		
3			6			6		1	5			1	4	1				6			6	
4			6			6		2	4			2	4		1	5					2	4
5	1	5				6			6			6					6				5	1
6			6		3	3			6				5	1			6				6	
7			6	1	2	3			6		4	2			1	5					6	
8			6		2	4	1	5				6				6					6	
9			6			6	2	4				6					4	2				6
10		6				6		1	2	4		6						6			6	
Total	1	11		1	7		3	25	29		6	39	13		2	16	23		2	10	37	

TABLE II

EFFECT OF VARYING TIME BETWEEN SENSITIZATION AND CHALLENGE

Animal	*Erythema Ratings* *Weeks between Sensitization and Challenge*											
	1			*2*			*3*			*4*		
	±	*Tr.*	0	±	*Tr.*	0	±	*Tr.*	0	±	*Tr.*	0
1	1	5			6				6			6
2		2	4		3	3			6			6
3		5	1	4	2				6			6
4		6		3	3				6		1	5
5			6		2	4			6	1	5	
6	6				2	4			6			6
7	1	2	3	2	4			4	2			6
8	5	1				6	3	3				6
9	6					6			6			6
10	6				1	5		6				6
Total	25	21		9	23		3	13		1	6	

Sensitization effects had almost disappeared by the fourth week.

Relative Effectiveness of Sensitization via Ears and via Flanks Table III shows that the degree of sensitization achieved by placing material on the ears is nearly as good as that achieved by flank sensitization.

TABLE III

RELATIVE EFFECTIVENESS OF SENSITIZATION VIA THE EARS AND VIA THE FLANKS

	Ear Sensitized				*Flank Sensitized*			
	+	±	*Tr.*	0	+	±	*Tr.*	0
Total erythema ratings	3	17	14	2	6	13	14	3

Effect of Continuous Application If standard sensitizer is applied to the flanks once a day, day after day (except week-ends), the erythema (Table IV) tends to be slower to form than is the case with an ear-flank test and it tends to fade after a time. At no time during the test does it appear that results are obtained which would be more useful than those obtained in an ear-flank test.

TABLE IV

EFFECT OF CONTINUOUS APPLICATION TO THE FLANKS

Day of Application	*Erythema Rating*					
	Animal 1	*Animal 2*	*Animal 3*	*Animal 4*	*Animal 5*	*Animal 6*
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
6	0	0	0	0	0	0
7	Tr.	0	0	0	0	0
8	Tr.	+	0	0	0	0
9	Tr.	Tr.	0	0	Tr.	Tr.
10	±	±	0	0	+	±
13	+	Tr.	0	0	+	+
14	±	Tr.	0	0	++	+
15	++	±	Tr.	0	++	++
16	+	+	Tr.	0	+	++
17	+	±	0	±	+	±
20	+	0	0	Tr.	+	+
21	Tr.	0	0	Tr.	±	+
22	Tr.	±	0	Tr.	±	+
23	Tr.	0	0	0	±	+

Relation between Concentrations of Sensitizing and Challenge Solutions and the Sensitization Induced Results in Table V indicate that concentrations of both sensitizing and challenge solutions are important in defining the final erythema obtained. They indicate also that, in a random test for sensitizing ability, positive results could be missed if too weak a solution were used. To avoid this, material in a test can be applied at the sensitizing stage in a concentration limited only by solubility. At the challenge stage a range of non-irritant concentrations can be applied to gain knowledge of the level of sensitization induced.

TABLE V

EFFECT OF ALTERING CONCENTRATIONS OF SENSITIZING AND CHALLENGE SOLUTIONS

Sensitization Concentration	*Challenge Concentration*		
	0·05%	0·25%	1·0%
0.05%			
Animal 1	Tr.	±	++
Animal 2	0	Tr.	++
Animal 3	Tr.	Tr.	±
0·25%			
Animal 1	0	±	++
Animal 2	0	+	++
Animal 3	Tr.	+	++
1·0%			
Animal 1	0	+	++
Animal 2	0	++	++
Animal 3	0	+	++

Frey and Wenk (1957), working with acetone solutions of DNCB, found that raising the concentration of DNCB from 0·3% to 0·9% raised the proportion of animals sensitized from 10% to 90%.

Effect of Injections of Freund's Adjuvant If Freund's adjuvant is injected alongside a second weaker sensitizer, either intraperitoneally or into the foot-pad of the guinea-pig, the immunological response to the second sensitizer is greater than if the second sensitizer had been injected alone (Landsteiner and Chase, 1940). To see if the results of a sensitization by standard sensitizer are affected by injections of adjuvant, guinea-pigs were given foot-pad injections of complete Freund's adjuvant before ear sensitization with DNCB, and the resulting erythema was compared with that of the controls. Table VI shows that the final erythema produced was unaffected by the injection of Freund's adjuvant at a site remote from the sites of treatment with DNCB.

TABLE VI

EFFECT OF FREUND'S ADJUVANT TREATMENT ON DEGREE OF SENSITIZATION

Treated with Freund's Adjuvant							*Untreated Controls*						
Guinea-pig	*Result*						*Guinea-pig*	*Result*					
1	±	±	±	±	±	±	5	o	o	o	Tr.	Tr.	o
2	±	±	±	o	o	Tr.	6	±	±	±	+	+	+
3	o	Tr.	o	o	o	o	7	Tr.	o	o	o	o	Tr.
4	o	o	o	Tr.	Tr.	Tr.	8	Tr.	±	±	±	±	±

Use of Animals other than the Guinea-pig Albino Syrian hamsters, albino mice, albino Wistar rats, and New Zealand white rabbits showed no sensitization effects with solutions of DNCB in olive oil using the usual test conditions. Rabbit skin seemed to give a primary irritant response more readily than guinea-pig skin. An irritant effect was seen in the rabbit, for instance, with 1% DNCB in olive oil, whereas the guinea-pig was unaffected by DNCB at this concentration. Rhesus monkeys (*Macaca mulatta*) could not be sensitized by applications of 5% DNCB in olive oil to the skin of the back or the groin.

Effect of Solvent The effect of solvent on the degree of erythema produced by standard sensitizer was investigated using a range of solvents which are known not to sensitize. The results shown in Table VII indicate that there is some variation in the degree of sensitization depending on the solvent chosen. The least satisfactory sensitizations were obtained with acetone, propylene glycol, glycerine, and ethanol solutions; moderately satisfactory sensitizations were achieved using dinonyl phthalate, olive oil, and paraffin oil solutions; and the most satisfactory sensitizations were achieved using dimethylformamide solutions. Tween 80, which gave rise to the most pronounced erythema, also gave some effect in controls.

TABLE VII

EFFECT OF SOLVENT ON SENSITIZATION PRODUCED BY DNCB

Solvent	*Total Erythema Ratings (6 per animal)*			
	++	+	±	Tr.
Olive oil		6	9	21
Dimethyl formamide ..		11	23	
Dinonyl phthalate		5	17	14
Acetone			12	24
Ethyl alcohol			29	7
Liquid paraffin		5	8	23
Propylene glycol		1	11	24
Glycerine			30	6
Tween 80[1]	12	13	5	2

[1]Some slight erythema was induced by this solvent in the controls.

PART II

Test of Chemicals for Sensitization

Method In testing chemicals for sensitizing ability the standard ear-flank test has been used. The test substance, in a suitable vehicle, is applied daily to the outer surface of the ears (0·1 ml. per ear per day) of Alderley Park strain albino guinea-pigs for three days, then on the seventh day after the start of the test a range of concentrations of solutions (0·2 ml.) of test material is applied to 1 cm. diameter circular areas on the clipped flanks of the guinea-pigs. The erythema produced is rated 24 hours later. Solutions of test material are also applied to the clipped flanks of control animals which have had no previous treatment on the ears. Only erythema arising in pretreated animals is considered to denote sensitization; that also arising in the controls denotes simple irritation. Guinea-pigs are used for this test about two months after birth and are reared on a standard diet consisting of barley 40 parts (by weight), Sussex ground oats 12·5 parts, middlings 15 parts, linseed cake 7·5 parts, white fish meal 7·5 parts, grass meal 15 parts, and ascorbic acid, vitamin, and mineral supplements to 100 parts, with vitamin C supplement in the drinking water. The animals are housed individually from weaning so that their skins are free from scratches. Immediately prior to application of the solutions, clipping is performed as close to the skin as possible using an Oster small-animal clipper with a no. 80 size 40 blade.

The ear-flank test has three advantages: (*a*) the painting of the ears instead of the flank is quicker, since no clipping or shaving is required; (*b*) the flanks when finally clipped are free of previous contact with the sensitizer; and (*c*) material applied to the ear appears in most cases not to be interfered with by the the guinea-pig and so the use of occlusive dressings can be avoided. Tests for sensitization in guinea-pigs with material applied percutaneously and thereafter protected with dressings tend to give results different from those in which material is applied to the skin without cover, because the hydration of the skin under the dressing makes the skin more permeable.

Results A wide range of industrial chemicals, including some known or suspected to give rise to skin sensitization and including some known from long usage to be non-sensitizing, has been looked at in the ear-flank test in guinea-pigs. Most materials produced no effect and, in fact, most irritant substances did not sensitize. Table VIII shows examples of cases where sensitizing potential has been detected by the test, and Table IX shows examples of compounds not producing sensitization under the conditions used. In Table VIII the substances have been classified by the chemical groupings to which they belong. The classification system is not intended to imply that all other compounds belonging to these groups are likely to be strong sensitizers.

Groups of substances showing sensitization effects can be classified as follows:

Activated Aryl Halides Various simple halogenated aromatic compounds, such as trichlorotrifluorobenzene, and halogenated aromatic compounds possessing only one nitro group, such as 2-chloro-4-nitroaniline, do not give rise to sensitization in the test (see Table IX). In order to sensitize, it appears that at least two nitro groups have to be present in an aryl halide, although Munn (1966) considers that *o*- and *p*-nitrochlorobenzene can sensitize humans. The relationship between activity and structure in this class of sensitizers has been discussed by Landsteiner and Jacobs (1935). Goldblatt (1945) noted that certain chlorosubstituted heterocycles, such as 2,4,6-trichloropyrimidine and 2,4,6-trichlorotriazine (cyanuric chloride), are powerful human vesicants, and that the former compound at least is hypersensitizing. Cyanuric chloride has been stated by Munn (1966) to sensitize humans.

Activated Aromatic Nitro Compounds Landsteiner and Di Somma (1940) have pointed out that such aromatic nitro compounds as 1,2,4-trinitrobenzene contain nitro groups which are replaceable and that for this reason such compounds are able to sensitize. 4-Nitroquinoline *N*-oxide likewise contains a replaceable nitro group and likewise sensitizes (Table VIII). This substance is also a skin carcinogen (Takayama, 1960).

Isocyanates Sensitization of guinea-pigs by aryl mono- and poly-isocyanates appears to be a general property of this class of compounds. Zapp (1959) has also noted that 2,4-tolylene diisocyanate sensitizes guinea-pigs. Since these compounds are considered to be inhalation sensitizers, they are handled carefully and not allowed to come into contact with chemical operatives. It is perhaps because of this and because isocyanates only demonstrate sensitization in animals on being applied in special unreactive vehicles such as dinonyl phthalate that skin sensitization by these compounds in humans has only occasionally been reported (Chief Inspector of Factories, 1956).

Isothiocyanates and Thiocyanates Aryl isothiocyanates have reactivity comparable to that of the isocyanates, and so it is not surprising that they are shown to be sensitizers in the guinea-pig test (Table VIII). Eisen and Belman (1953) showed that 2,4-dinitrophenyl thiocyanate sensitized guinea-pigs.

The typical alkyl isothiocyanate, allyl isothiocyanate, has been shown to be a sensitizer in human beings and in pigs but not in monkeys, rabbits, and guinea-pigs (Landsteiner and Di Somma, 1938). α-Methylallyl isothiocyanate (compound 57, Table IX) also fails to sensitize guinea-pigs. Goldblatt (1945) has reported that organic isothiocyanates are strong skin vesicants in humans and that hypersensitivity frequently follows skin contact.

Aromatic Hydrazines Aromatic hydrazines appear to be sensitizers irrespective of whether or not the aromatic ring also has activating nitro substituents (see Table VIII). It has been reported that 4-chloro-2-methylphenylhydrazine is a strong human sensitizer (Malten, 1958) and that phenyl hydrazine sensitizes small animals (Jadassohn, 1930). Wheeler, Penn, and Cawley (1965) found that workers exposed to hydrazine develop a contact dermatitis and are then cross-sensitive to hydrazinophthalazine and to phenylhydrazine.

Aminodiphenyls Since some workers (Old, Benacerraf, and Carswell, 1963; and Gordon, 1964) have speculated that some of the basic processes of sensitization and carcinogenesis may involve similar

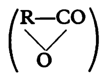
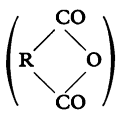
TABLE VIII

COMPOUNDS CAUSING SENSITIZATION

Compounds[1]	*No. of Animals Tested*	*Solution Applied in Sensitization and Challenge Stages*	*Erythema Rating*[2] ++	+	±	Tr.
Activated aryl halides (ArHal)						
2, 4-Dinitrochlorobenzene	12	0·25% and 0·25% in olive oil[3]			5	6
Picryl chloride	8	10% and 10% in olive oil		1	7	
Cyanuric chloride	8	10% and 1% in DMF	1	4	1	2
Activated aromatic nitro compounds						
4-Nitroquinoline *N*-oxide	5	10% and 0·05% in DMF	3	2		
Isocyanates (RNCO)						
2, 4-Tolylene diisocyanate	8	1% and 1% in DNP	1	2	2	3
2, 6-Tolylene diisocyanate	10	1% and 1% in DNP	2	3	4	1
o, *p*′-Diisocyanatodiphenylmethane	4	1% and 1% in DNP	1	3		
o-Tolyl isocyanate	6	10% and 0·5% in DNP		2	3	1
Phenyl isocyanate	6	10% and 0·5% in DNP		1	3	2
Isothiocyanates and thiocyanates (RNCS, RSCN)						
Phenyl isothiocyanate	8	10% and 10% in DNP			4	4
2-Amino-6-thiocyanatobenzthiazole	8	10% and 10% in DMF			3	1
Aromatic hydrazines ($ArNHNH_2$)						
Phenylhydrazine	8	10% and 10% in DNP	1	1	5	1
4-Chloro-2-methylphenylhydrazine	12	10% and 10% in DMF	1	9	2	
2, 4-Dinitrophenylhydrazine	8	10% and 10% in DMF	1	5	1	1
2, 4-Dichlorophenylhydrazine	8	10% and 10% in DMF		6	2	
Aminodiphenyls (NH_2ArArR)						
Benzidine	8	10% and 10% in DMF		1	2	3
Phenylene diamines and aminodiphenylamines (NH_2ArNH_2 and $NH_2ArNHAr$)						
p-Phenylene diamine	8	10% and 1% in DMF		8[4]		
p-Aminodiphenylamine	4	1% and 1% in olive oil			3	1
o-Aminodiphenylamine	6	10% and 1% in DMF			5	1
Nitroamines ($NO_2.NHR$)						
Ethylene *bis*-nitrourethane	4	0·5% and 0·5% in DNP			2	2
Nitrosoamines (NO.NHR)						
Nitrosomethylurea	6	10% and 10% in DMF	1	5		
N-Methyl-*N*′-nitro-*N*-nitrosoguanidine	8	10% and 1% in DMF		3	5	
Active aralkyl halides ($ArCH_2.Hal$)						
2, 4-Diamino-6-chloromethyl-*s*-triazine	10	2% and 2% in DMF			5	3
2, 4-Diamino-6-bromomethyl-*s*-triazine	10	2% and 2% in DMF		4	5	1
p-Chlorobenzyl chloride	6	10% and 10% in DMF			2	
Halogenated aryl ketones ($ArCO.CH_2.Hal$)						
f-Bromophenacyl bromide	6	10% and 1% in DMF		2	4	
Epoxy compounds ($R\,\overbrace{CH-CH}^{O}R$)						
2, 3-Epoxypropylphenyl ether	6	10% and 10% in olive oil			2	4
Styrene oxide	8	10% and 10% in olive oil			1	5
Carbodiimides (RN:C:NR)						
N, *N*′-Dicyclohexylcarbodiimide	8	10% and 0·25% in DNP			2	4
Nitrosoaromatic compounds (ArNO)						
p-Nitrosodimethylaniline	6	10% and 10% in DMF	2	2	2	
6-Nitroso-3-cresol	6	10% and 1% in DMF		2	4	
p-Nitrosodiphenylamine	4	10% and 0·5% in DMF	2	2		

(cont.)

TABLE VIII (continued)

Compounds[1]	*No. of Animals Tested*	*Solution Applied in Sensitization and Challenge Stages*	*Erythema Rating*[2]			
			++	+	±	Tr.
Activated phenols (ArOH)						
2, 4, 5-Trichlorophenol	8	10% and 10% in DMF			2	5
Pentachlorophenol	8	5% and 5% in olive oil			2[5]	
Alkylating agents						
Diazomethane	8	0·3% and 0·3% in 50% ether/olive oil				6
Aroyl halides (ArCO.Hal)						
3, 5-Dinitrobenzoyl chloride	6	10% and 10% in DMF				6
Aryl sulphonhalides ($ArSO_2$.Hal)						
Toluene sulphonylchloride	6	10% and 5% in DMF			5	
Aryl sulphenyl halides (ArSHal)						
2, 4-Dinitrophenylsulphenyl halides	6	10% and 1% in DMF		2	2	3
Active lactones 						
β-Propiolactone	6	100% on ears, 2% in 50% H_2O/DMF challenge		2		
Active anhydrides 						
Phthalic anhydride	8	10% and 5% in DMF		4	3	1[6]
Maleic anhydride	9	20% and 1% in DMF		4	2	1
Active aromatic esters ($ArCO_2R$)						
Dimethylaminoethyl *p*-nitrobenzoate	8	10% and 10% in DMF			2	4
Dimethylaminoethyl 3, 5-dinitrobenzoate	8	10% and 10% in DMF			1	2
Aldehydes (RCHO)						
Formaldehyde	8	10% formalin in water, challenge 5% formalin in 50% aqueous DMF			5	
Metal anions and cations						
Nickel chloride	6	10% in olive oil:Tween 80 (1:1) challenge 2% in olive oil:Tween 80 (9:1)		1	5	

[1]In formulae ArHal, RNCO, etc., Ar stands for a substituted or unsubstituted aromatic (aryl) group such as phenyl, naphthyl, thiazolyl, thienyl, furyl, etc.; R stands for alkyl or aryl; and Hal stands for halogen.
[2]Ratings are on the scale ++ (bright pink), + (pink), ± (light pink), Tr. (just observable erythema).
[3]Standard sensitizer test.
[4]Read after BaS depilation.
[5]All other animals and controls gave a trace reaction which was concentration independent.
[6]Some lesser reaction was seen in controls.
DMF — dimethyl formamide.
DNP — dinonyl phthalate.

processes, involving reactive compounds combining with proteins to give abnormal proteins, it is interesting that benzidine has both carcinogenic (Spitz, Maguigan, and Dobriner, 1950) and sensitizing properties (Table VIII).

Definite cases of sensitization of humans to aminodiphenyl compounds do not appear to have been reported, though benzidine is included in a list of compounds (Schwartz, Tulipan, and Birmingham, 1957) stated to be dermatitic and in a list of compounds (Schwartz, 1943) stated to be sensitizers. Munn (1966) has noted no cases of sensitization to pure benzidine among a large number of dyestuffs workers concerned with the manufacture of this compound.

TABLE IX

COMPOUNDS NOT CAUSING SENSITIZATION[1]

No.	*Substance*	*No. of Animals Tested*	*Solution*
1	f-Dichlorobenzene	5	10% in DMF
2	Nitrobenzene	6	10% in DMF
3	Epichlorohydrin	8	10% in olive oil
4	Aniline	8	20% and 10% in DMF
5	Anthracene	5	5% in DMF
6	Naphthalene	8	10% in DMF
7	Phenanthrene	4	10% in DMF
8	Phenol	7	5% in DMF
9	Pyridine	8	10% in DMF
10	Fluorene	4	10% in DMF
11	2-Chloro-4-nitroaniline	8	10% in DMF
12	4-Chloro-2-toluidine	8	10% in DMF
13	Quinhydrone	8	10% in DMF[2]
14	4-Nitrophenoxyethyl bromide	8	10% in DMF
15	Pyrogallol	8	10% in DMF
16	4-Anilinoazobenzene	8	10% in DMF[3]
17	4-Chloro-2-nitroaniline	8	10% in DMF
18	Phenylazo-2-naphthol	8	5% in DMF[3]
19	Nitroguanidine	6	10% in DMF
20	Ninhydrin	6	10% in DMF[3]
21	Dimethyl sulphate	6	10% in olive oil
22	3, 5-Dimethylmorpholine	6	1% in olive oil
23	Dichlorotetrafluorobenzene	4	5% in olive oil
24	Trichlorotrifluorobenzene	4	5% in olive oil
25	Nitrosodimethylamine	7	10% in olive oil
26	*N*-Nitrosopiperidine	12	10% in olive oil
27	*N*-Nitroso-*N*-methylaniline	12	10% in olive oil
28	Chloropentafluorobenzene	4	10% in olive oil
29	Neoarsphenamine[4]	8	10% in 50% DMF/H_2O
30	2-Amino-5-hydroxybenzoic acid	8	5% in DMF
31	2-Amino-5-chlorotoluene	8	10% in DMF
32	3-Nitro-4-toluidine	8	10% in DMF
33	2-Nitro-4-toluidine	8	10% in DMF
34	4-Nitro-2-toluidine	8	10% in DMF
35	f-Amino-*N*, *N*-diethyl aniline HCl	8	10% in 50% DMF/H_2O
36	Methyl thioglycollate	8	10% in DNP
37	Chloroacetyl chloride	8	10% in DNP
38	Potassium chromate	8	2·5% in 50% DMF/H_2O
39	4, 4′-Dipyridyl	12	5% in DMF
40	*N*-Chloroacetyldimethylmorpholine	12	5% in DMF
41	Menazon	6	10% in DMF
42	Azodicarbonamide	4	1% in DMF
43	*N*-Bromosuccinimide	4	10% in DMF
44	2, 6-Dihydroxynaphthalene	6	10% in DMF
45	2, 3-Dihydroxynaphthalene	6	10% in DMF
46	Tribenzylamine	6	10% in DMF
47	1-Amino-4-naphthol	6	10% in DMF
48	2, 6-Dichloro-4-benzoquinone 4-chlorimine[5]	6	10% in DMF
49	*p*-Nitrobenzoyl chloride	6	10% in DMF
50	1, 4-Dihydroxyanthraquinone	6	5% in DMF

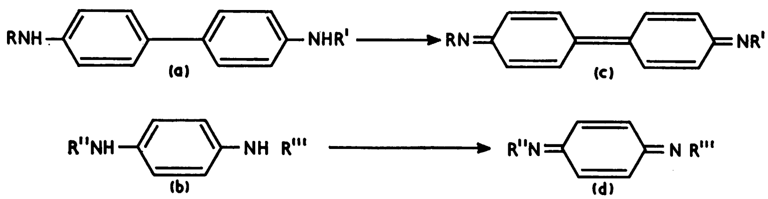
(cont.)

TABLE IX (continued)

COMPOUNDS NOT CAUSING SENSITIZATION[1]

No.	Substance	No. of Animals Tested	Solution
51	Girard's reagent	6	10% in DMF
52	Acetal	6	10% in olive oil
53	2-Oxazolidone	8	10% in DMF
54	1, 1-Carbonyldiimidazole	8	10% in DMF
55	1, 4-*bis*-Dicyanoethylpiperazine	8	10% in DMF
56	*p*-*N*-Isopropylamino diphenylamine[4]	4	5% in olive oil
57	α-Methylallyl isothiocyanate	8	10% in DNP
58	Nitroarginine	8	10% in DMF

[1]Under the conditions of test tried.
[2]Skin rendered dark black.
[3]Skin rendered orange by substance.
[4]This substance has been shown (Frei, 1928) to sensitize in certain circumstances.
[5]This substance rendered the skin yellow-black.
[6]No erythema is seen 24 hours after challenge, but strong erythema is seen after 48 hours; such reactions are seen, however, on controls.



It is thought (Landsteiner and Jacobs, 1935; and Mayer, 1954) that aromatic amines of this type (a) and those belonging to the class of substituted phenylene diamines (b) and amino-diphenylamines (b, R'' = phenyl) only sensitize after metabolism to reactive iminoquinoid forms (c and d).

Phenylene Diamines and Aminodiphenylamines *p*-Phenylene diamine, a dye used in the fur trade, has long been known to sensitize humans (Mayer, 1929) and guinea-pigs (Mayer, 1954). *p*-Amino-diphenylamine sensitizes guinea-pigs strongly (Table VIII), but *N*-alkyl derivatives thereof (substance 56, Table IX) do not appear to sensitize under the usual conditions of test. Munn (1966) considers *p*-aminodiphenylamine to be a human sensitizer.

Nitroamines Ethylene-*bis*-nitrourethane and related derivatives sensitize guinea-pigs (Table VIII). They have also shown the ability to sensitize (Mees, 1966) workers involved in research (Borer, Hardy, Lindsay, Spratt, and Mees, 1966) on these compounds. The sensitizing potential of these *N*-nitroethylene diamine compounds has been observed also by Davies (1964b). Sensitizing activity does not seem to be general to this type of compound since nitroguanidine and nitroarginine (substances 19 and 58, Table IX) do not sensitize guinea-pigs.

Nitrosoamines *N*-Methyl-*N'*-nitro-*N*-nitroso-guanidine is a powerful mutagen (Adelberg, Mandel, and Chen, 1965). Its action as a skin sensitizer (Table VIII) in the guinea-pig may be due to its *N*-nitro or its *N*-nitroso groups, or to both. *N*-Nitrosomethylurea, which may sensitize the guinea-pig by conversion *in vivo* to diazomethane, has been reported by Rosen (1953) to give rise in humans to a poison ivy-type rash, and by Druckrey, Steinhoff, Preussmann, and Ivankovic (1964) to be carcinogenic in the rat. Nitrosomethylurethane sensitizes man (Fleming, 1960) and is carcinogenic in the rat (Schoental, 1963). *N*-Nitrosodimethyl-amine, *N*-nitrosopiperidine, and *N*-nitroso-*N*-methyl aniline (substances 25, 26, and 27, Table IX), members of a series some of which are potent liver carcinogens (Druckrey, Preussmann, Schmähl, and Müller, 1961), do not appear to sensitize under the test conditions tried.

Active Aralkyl Halides Such active aralkyl halogen compounds as *p*-chlorobenzyl chloride and 2,4-dinitrobenzyl chloride are known (Landsteiner and Jacobs, 1936) to sensitize guinea-pigs. The demonstration that halogenomethyltriazines (Table VIII) are powerful sensitizers shows that this activity is not confined to homoaromatic systems. These triazines have in addition shown very powerful sensitizing action against laboratory workers handling them (Whitehead, 1962).

Halogenated Aryl Ketones Compounds belonging to the group of ω-halogenoacetophenones such as *p*-bromophenacyl bromide (Table VIII) are well known for their lachrymatory effect. 2,4,6-Trimethylphenacyl bromide (Sloan-Kettering Institute, 1953) has been described as a sensitizer.

Epoxy Compounds It has been known for some time that the epoxy components used in the preparation of epoxy resin have the power to sensitize humans (Malten and Zielhuis, 1964), but these compounds do not appear to have been thoroughly studied in animal tests. They do certainly sensitize the guinea-pig (Table VIII). Fregert and Rorsman (1964a) have stated that, of 20 patients with contact allergy to epoxy resins, 14 reacted to a patch test with phenylglycidyl ether (2,3-epoxypropylphenyl ether), three to butylglycidyl ether, and two to allyl glycidyl ether. Epichlorohydrin (compound 3, Table IX) did not sensitize guinea-pigs under the conditions studied.

Carbodiimides Compounds with this type of structure have only recently been used in laboratory synthetic work and therefore sensitization of humans to these compounds is unlikely to have occurred as yet. Although dicyclohexyl carbodiimide, the member of the series most used in laboratory work, sensitizes guinea-pigs (Table VIII), carbonyl diimidazole (substance 54, Table IX) appears not to do so under the conditions tried.

Nitrosoaromatic Compounds The strong sensitizing power to guinea-pig skin of compounds in this series was noted by Landsteiner and Jacobs (1935). *p*-Nitrosodimethylaniline was particularly powerful, but activity was also observed in *p*-nitrosophenol. Experimental induction of sensitization to *p*-nitrosodimethylaniline has been achieved in man (Meneghini, 1963). *p*-Nitrosodiphenylamine is considered (Munn, 1966) to sensitize humans and certainly sensitizes guinea-pigs (Table VIII).

Activated Phenols Most phenols, including phenol itself (compound 8, Table IX), even though they are irritant, do not cause sensitization. Since the phenol group would not be expected to link covalently to proteins, it would not be thought that any phenol would have the power to sensitize. It appears, however, that certain active phenols such as picric acid are sensitizers (Landsteiner and Di Somma, 1940) since they are able to form antigen conjugates by strong ionic bonding with proteins. Landsteiner and Jacobs (1935) also found sensitizing power to the guinea-pig in 2,4-dinitrophenol, f-aminophenol, and *m*-chloracetylaminophenol. Dinitrophenol is reported to be a sensitizer in humans (Fleming, 1960).

Alkylating Agents Methylating agents, such as diazomethane, were shown by Landsteiner and Di Somma (1938) to sensitize guinea-pigs though another methylating agent, dimethyl sulphate, gave an irregular reaction (see substance 21, Table IX). Sime nitroso compounds which can decompose to diazomethane (or its homologues) also sensitize and may possibly belong to this group. The alkylating agent, dichloroethyl sulphide, is said to sensitize man (Fleming, 1960).

Aroyl Halides The ear-flank test gives a weakly positive result (Table VIII) with the acid chlorides of certain aromatic acids. If other acid chlorides, such as *p*-nitro-benzoyl chloride, have any sensitization effect it is so marginal that it is not detected in the ear-flank test (compound 49, Table IX). Landsteiner and Jacobs (1936) were in fact only able to demonstrate sensitization of guinea-pigs if *p*-nitrobenzoyl chloride was injected intradermally to by-pass the skin barrier. The failure of certain simple acid chlorides, such as chloro-acetyl chloride (compound 37, Table IX), to sensitize is possibly due to the rapid rate of reaction of this compound with moisture on the skin.

Aryl Sulphonhalides *p*-Bromobenzene sulphonyl chloride and *m*-nitrobenzene sulphonyl chloride (Landsteiner and Jacobs, 1936) as well as toluene sulphonyl chloride (Table VIII) sensitize guinea-pigs.

Aryl Sulphonyl Halides 2,4-Dinitrophenylsulphenyl chloride can be shown (Table VIII) to sensitize guinea-pigs. Sensitization of guinea-pigs and man to this compound was reported by Eisen and Belman (1953).

Active Lactones β-Propiolactone is of particular interest because it shows carcinogenic potential as well as sensitizing potential (Table VIII). It is a skin carcinogen in both the mouse (Palmes, Orris, and Nelson, 1962) and the guinea-pig (Parish and Searle, 1966).

Active Anhydrides Guinea-pigs are sensitized by citraconic anhydride (Hunziker, 1964) and by phthalic and nitrophthalic anhydrides (Jacobs, Golden and Kelley, 1940). Phthalic anhydride gives rise to dermatitis in humans (Kito and Tosu, 1953) but any sensitization effect involved has been attributed to naphthoquinone impurity.

Active Aromatic Esters Certain esters of *p*-nitrobenzoic and 3,5-dinitrobenzoic acids are found (Table VIII) to sensitize guinea-pigs. Procaine (2-diethylaminoethyl 4-amino-benzoate) is known to sensitize humans.

Aldehydes Formaldehyde, a substance which is known to sensitize humans (Eberhartinger and Ebner, 1964), can be shown to sensitize the guinea-pig (Table VIII). Substances with a latent aldehyde function, such as an acetal, might also belong to this class. The monoacetal, 2-phenyl-4-ethoxymethylene-oxazolone, has been shown (Gell, Harington, and Michel, 1948) to sensitize guinea-pigs.

Metal Anions and Cations Table VIII shows that the sensitization effect of nickel ion can be demonstrated in the guinea-pig using nickel ion in olive oil-Tween 80. Nickel (Duperrat and Lamberton, 1962) as well as cobalt (Geiser, Jeanneret, and Delacretaz, 1960), beryllium (Curtis, 1951), and chromate (Fregert and Rorsman, 1964b) are known to sensitize humans. Potassium chromate (compound 38, Table IX) did not sensitize guinea-pigs under the conditions tried.

Discussion

A rapidly performed percutaneous test for skin sensitization in the guinea-pig is an attractive test for detecting strong sensitizers among industrial chemicals. Under a standard set of conditions, such as is approached in the test used in this work involving application to the ears and then to the flank skin, a roughly quantitative comparison can be made between any sensitizer and a standard sensitizer such as DNCB. This is achieved by comparing the concentrations of the test material and of the standard allergen which give equivalent sensitizations in a statistically significant number of guinea-pigs. The one condition of the test which cannot be standardized is the vehicle in which the sensitizer is applied. Olive oil or other bland oil is most desirable but either insolubility (cf., 2,4-diamino-6-chloromethyl-*s*-triazine) or reactivity (cf., tolylene diisocyanate) may require the use of other vehicles and there is no doubt that the vehicle does influence the degree of erythema obtained.

Although tests for sensitizing potential involving intradermal injections at the sensitizing and/or challenge stages tend to give positive results with more compounds than does a percutaneous test, there is some doubt about the usefulness of such a test. Doubt about the value of intradermal tests has also been expressed by Rowe and Olson (1965). When testing for sensitizing potential in a drug, such intradermal (Draize, 1959) techniques have undoubted value because humans are exposed to drugs given by a whole variety of means. But such an intradermal test as the Draize test (Witjens, 1964) may fail to detect such an obvious sensitizer as DNCB. Skin sensitization to industrial allergens is almost always a percutaneous process, or at least the challenge is percutaneous, unless some special situation applies, as when the skin is scuffed or cut. An illustration of a case where sensitization potential has been shown only by intradermal testing and apparently does not show with percutaneous testing is ethylene dinitroamine (EDNA). This compound is closely related to ethylene-*bis*-nitrourethane, an olive oil solution of which shows up as a strong sensitizer in the ear-flank test. Ethylene dinitroamine, being an ionic compound, is not soluble in oils, but only in water. That ethylene dinitroamine has the same ability as the related urethane to form an antigenic conjugate is shown by injecting this material in saline solution intradermally into the ears on three days and then into the flanks on the seventh day. As far as is known, however, in any case of human exposure the compound giving rise to sensitization in a percutaneous test against guinea-pigs is the one giving rise to human sensitization and not the compound giving sensitization in an intradermal test. Various workers, including Somers (1964), have strongly questioned the value of sensitization testing in guinea-pigs and feel that patch testing of humans gives more useful information, but it has been pointed out by Philp (1964) that a guinea-pig test will show up the sensitizing power of a known human sensitizer, for instance that of nickel, which a patch test on humans will fail to demonstrate. Other workers, notably Maibach and Epstein (1965), have pointed out that the results of patch testing on humans are seldom as easy to interpret as is commonly supposed.

The reaction observed on the skin of a sensitized guinea-pig following challenge takes 24 hours to reach maximum intensity and is called a reaction of delayed hypersensitivity to differentiate it from one in which an erythema reaction builds up more rapidly, say one-quarter to four hours, which is called immediate hypersensitivity. The biological factors which are concerned in the development of this delayed hypersensitivity in guinea-pigs have been the subject of much study (Frey and Wenk, 1957; Landsteiner and Chase, 1942; Chase, 1945). From these studies it is apparent that (*a*) the influence of sensitizing substance applied to the skin is transmitted to the regional lymph nodes via the lymph vessels; (*b*) the nodes are indispensible for the development of sensitization but other areas are also involved; (*c*) the effect of the sensitization returns to the skin via the blood stream; and (*d*) sensitization can be transferred to another animal by transfer of lymphocytes. The relation of delayed hypersensitivity and contact sensitivity in the guinea-pig has been further discussed by Salvin and Smith (1961). Despite the considerable progress made in understanding the process by which delayed skin sensitivity develops in the guinea-pig, our understanding of the relationship between this process and the process occurring in humans during development of contact sensitization is still incomplete.

Finally, it is necessary to consider the usefulness of negative results in the guinea-pig test. The value of a negative result must be limited, since certain weak sensitizers, *i.e.*, sensitizers affecting only a small proportion of the people with whom it comes in contact, fail to be detected by such a test. It cannot, therefore, be concluded that compounds showing no sensitizing effect in the guinea-pig will not sensitize some humans. It is important in this connexion to point out, however, that a large-scale skin test on humans, which is presently the only other test for sensitizers, can also fail to detect certain weak sensitizers (Philp, 1964). A negative result in the guinea-pig test may be useful, however, in predicting that an industrial chemical is not a strong sensitizer, *i.e.*, a sensitizer affecting most people with whom it comes in contact. If general plant experience suggests that a particular chemical is not a strong sensitizer to humans and this impression is reinforced by tests in guinea-pigs, there would be reason to believe that a positive patch test found in most of the plant operatives involved may be wrongly interpreted or due to impurities in a chemical sample tested. The claims that such substances as dichlorobenzene and nitrobenzene (Fleming, 1960) and such substances as aniline, anthracene, naphthalene, phenanthrene, phenol, pyridine, fluorene, and lime (Schwarz, 1943) are sensitizers to humans would, for instance, deserve re-examination in the light of the fact that these compounds do not sensitize in a guinea-pig test.

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